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(54) Title: SYSTEM FOR THE RAPID MANIPULATION OF NUCLEIC ACID SEQUENCES (54) Titre: SYSTEME SERVANT A MANIPULER RAPIDEMENT DES SEQUENCES D'ACIDES NUCLEIQUES		
(57) Abstract		
<p>The present invention is a cell-free subcloning system utilizing three elements: (1) a donor vector that contains a nucleic acid sequence to be transferred to another vector flanked by a site-specific recombination sequence and one or more optional additional nucleic acid sequences, (2) an acceptor vector that contains a site-specific recombination sequence and one or more optional additional nucleic acid sequences, and (3) a site-specific recombinase that recognizes the site-specific recombination sequences in the donor and acceptor vectors so as to transfer the transfer sequence from the donor to the acceptor vector upon contact of the three elements of the system. Also disclosed are rapid subcloning methods employing the vectors and enzymes disclosed herein and kits for use in such methods.</p>		
(57) Abrégé		
<p>L'invention concerne un système de sous-clonage exempt de cellules mettant en application trois éléments : (1) un vecteur donneur contenant une séquence d'acides nucléiques à transférer vers un autre vecteur flanqué par une séquence de recombinaison spécifique au site et une ou plusieurs séquences supplémentaires éventuelles d'acides nucléiques ; (2) un vecteur accepteur contenant une séquence de recombinaison spécifique au site et une ou plusieurs séquences supplémentaires éventuelles d'acides nucléiques, (3) une recombinaise spécifique au site reconnaissant les séquences de recombinaison spécifiques au site des vecteurs donneur et accepteur, de manière à transférer la séquence de transfert du vecteur donneur au vecteur accepteur par mise en contact des trois éléments du système. Elle concerne également des procédés rapides de sous-clonage mettant en application les vecteurs et les enzymes décrits, ainsi que des trousseaux d'utilisation de ces procédés.</p>		

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Description

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**SYSTEM FOR THE RAPID MANIPULATION
OF NUCLEIC ACID SEQUENCES**

Field of the Invention

5 The invention disclosed herein relates to the field of molecular biology and
10 methods useful therefor. More particularly the invention relates to methods for
15 subcloning of nucleic acid sequences.

Background of the Invention

20 The discovery and isolation of restriction endonucleases, specific enzymes
10 capable of manipulating nucleic acid sequences, precipitated a revolution in molecular
 biological techniques. Restriction endonucleases were used to cut large DNAs into
 smaller fragments that could be re-attached to heterologous pieces of DNA by ligases.
25 These techniques allowed scientists to transfer a gene encoding a particular protein
 into a relatively small plasmid vector that could be transfected into a cell for
15 production of the encoded protein.

30 Over the years, a large number of vectors have been developed for a wide
 variety of specialized research, manufacturing, and production uses. For example,
 many types of expression vectors have been developed that allow heterologous
35 proteins to be expressed in an increasingly larger number of cell types, including
20 insect, plant, mammalian, and bacterial cells. Among expression vectors, specialized
 vectors have been developed that facilitate large scale production of proteins, for
 instance, by increasing levels of the protein produced or by introducing elements into
40 the protein that aid in purification. Other vectors have been designed for use in
 specific research protocols, such as conducting one-hybrid or two-hybrid screens.
25 Each specialized vector contains a specific set of nucleic acid sequences that give it its
45 particular features. No one vector can contain all of these features, however, as the
 vector would eventually become too large to be easily manipulated. Thus, a nucleic
 acid sequence of interest must be moved from one vector to another as different
50 specialized needs arise, a process known as subcloning.

Conventional subcloning methods require that each vector into which a nucleic acid sequence is to be subcloned contain restriction endonuclease recognition/digestion sites that are absent in the nucleic acid sequence in order to prevent the nucleic acid sequence from being cut into one or more pieces when subjected to the restriction endonuclease for removal from the vector and passage to the next vector. One must, therefore, either know the entire sequence of the nucleic acid being subcloned or test it with each restriction endonuclease proposed for use to see if it contains a matching recognition site. Either process requires time and resources to perform.

In addition, conventional subcloning methods require that the nucleic acid sequence being subcloned have sequences at its 5' and 3' ends that match the restriction endonuclease site into which it is being inserted. As not all available vectors have the same restriction endonuclease sites, the nucleic acid sequence to be transferred must usually be modified at its ends to make it compatible with each vector to be used in subcloning techniques.

Another drawback to conventional subcloning techniques is the use of ligases. These enzymes are relatively slow acting, require ATP, and generally are highly temperature sensitive.

A need still exists in the art, therefore, for a simple, rapid system for the manipulation of nucleic acid sequences between vectors. The present invention addresses that need.

Brief Description of the Invention

The present invention comprises a cell-free subcloning system, methods for the rapid manipulation and subcloning of nucleic acid sequences using the system, and kits suitable for use in conducting such methods. In general, the system, methods, and kits of the invention utilize three elements. The first element is a donor vector comprising (1) a transfer sequence of nucleic acid to be transferred to an acceptor

5 vector, (2) a site specific recombination nucleic acid sequence flanking the transfer
sequence as shown in Figure 1A; and, (3) optionally, one or more additional nucleic
acid sequences. The second element is an acceptor vector comprising (1) a site-
10 specific recombination sequence that matches the site-specific recombination
5 sequence of the donor vector as shown in Figure 1B, and (2) one or more additional
nucleic acid sequences. The third element is a site-specific, ATP independent
15 recombinase, that recognizes the site specific recombination sequences in both the
donor and acceptor vectors.

The site-specific recombinases employed in the practice of the present
20 invention are enzymes that spontaneously recognize and cleave at least one strand of a
double strand of nucleic acids within a sequence segment known as the site-specific
recombination sequence. In the donor vector, the site specific recombination
25 sequences are placed contiguously on either side of (i.e., "flank") a transfer sequence
of nucleic acid whose excision from the donor vector and transfer to the acceptor
15 vector is desired. In use, the donor vector containing the transfer sequence and the
acceptor vector are placed within a single cell-free solution. Upon addition of the site-
specific recombinase to the cell-free solution, the transfer sequence is excised from
30 the donor vector. In some portion of the acceptor vectors in the cell-free solution (i.e.,
"occasionally") the excised transfer sequence is ligated into the acceptor vector by
35 20 operation of the recombinase upon the site-specific recombination sequence, without
the use of a separate ligase to accomplish the ligation. The acceptor vectors generally
further comprise a selectable marker gene to aid in identifying and isolating from the
cell-free solution using known methods those acceptor vectors into which the transfer
40 sequence has been successfully inserted. The site-specific recombination sequences
25 of the donor and acceptor vehicles are preferably identical, but can vary in nucleic
acid sequence so long as recognition of the site-specific recombination sequence by
the recombinase is preserved despite the variance.

The present invention thus affords a novel single-step method and associated
vectors and kits for moving nucleic acid sequences, such as recombinant DNA
50 30 molecules, from one type of subcloning vector to another that overcomes the above-

described problems in the art. For example, the invention eliminates the need for incorporation of "add on" base sequences to transfer sequence to provide unique restriction sites.

In particular, topoisomerase-based cloning circumvents any problems associated with addition of nontemplated nucleotides by DNA polymerase at the 3' end of the amplified DNA. Any nontemplated base (N) at the 3' end of a PCR product destined for topoisomerase-based transfer (GCCCTTxxxxN-3') will dissociate spontaneously upon covalent adduct formation, and will therefore have no impact on the ligation to vector. Second, the only molecule that can possibly be ligated into the acceptor vector is the covalently activated transfer sequence and the transfer sequence can only be transferred to the acceptor vector. There is no potential for in vitro covalent closure of the acceptor vector itself, which ensures low background. There is also no opportunity for the transfer sequences to ligate to one another, which precludes cloning of concatameric repeats. In addition, unintended internal restriction of an uncharacterized sequence is avoided because the use of common restriction enzymes is avoided.

Description of the Figures

FIGURE 1A is a double stranded nucleic acid sequence (SEQ ID NO:17 and complementary strand thereto) representing a donor vector with a double stranded nucleic acid transfer sequence flanked by topoisomerase I recombinase recognition sites (single underlined) with a 4 base core sequence (within brackets).

FIGURE 1B is a double stranded nucleic acid (SEQ ID NO:18 and complementary strand thereto) representing an invention acceptor vector containing two recombinase recognition sites that match those in the donor vector and 10 base pair spacer sequences (double underlined) ready to receive a transfer sequence.

FIGURE 1C is a double stranded nucleic acid (SEQ ID NO:19 and complementary strand thereto) representing a new recombinant vector created by the operation of topoisomerase I upon the donor and acceptor vectors of Figure 1A and 1B, respectively. The transfer sequence is now inserted into the acceptor vector.

FIGURE 2 is schematic representation of the method of the invention utilizing a donor vector ("pDonor") containing a selectable marker gene other than Zeocin, an origin of replication sequence ("ori"), and a transfer sequence ("gene of interest") flanked by lox P recognition sites. The acceptor vector ("pAcceptor") contains a gene encoding resistance to the antibiotic Zeocin™ ("Zeo"), an origin of replication sequence ("ori"), and a gene encoding ccdB, a lethal compound, flanked by loxP sites. The arrow indicates that when the donor and acceptor vectors are combined in a reaction mixture in the presence of the recombinase Cre, a new recombinant vector ("pRecombinant") is created, which recombinant vector contains the transfer sequence and a gene encoding Zeo. Cells transformed with the reaction mixture will grow in the presence of the antibiotic Zeocin™ only if the recombination event has successfully occurred.

Detailed Description of the Invention

In one embodiment of the invention, there is provided a cell-free subcloning system comprising (1) a donor vector comprising a transfer sequence flanked by site-specific recombination sequences, (2) an acceptor vector comprising a site-specific recombination sequence that matches the site-specific recombination sequences of the donor vector, and (3) a site-specific recombinase capable of recognizing the site-specific recombination sequence. Each vector is of duplex nucleic acid sequence, and the transfer is a bivalent strand transfer. In the acceptor vector, the transfer sequence will be inserted in the immediate vicinity of and downstream of, or can be adjacent to, the site-specific recombination sequence. Optionally, one or more additional nucleic acid sequences, such as a selection marker gene, an origin of replication, a promoter-enhancer sequence, and the like can be included in the donor and acceptor vectors.

5 The subcloning event occurs in a cell-free environment without the need to use restriction enzyme(s), and the transfer of the transfer sequence to the acceptor vector occurs without the expense of ATP.

10 In a presently preferred embodiment of the invention, following the site-specific recombination event that occurs between the site-specific recombination sequences located on each vector (i.e., the donor and acceptor vectors), the transfer sequence is inserted into the acceptor vector in a manner that retains the proper translational reading frame of the transfer sequence.

20 As used herein "vector" means a recombinant nucleic acid sequence of duplex DNA that has been constructed to comprise one or more functional units not found together in nature. Examples include circular, double-stranded, extrachromosomal DNA molecules (plasmids), cosmids (plasmids containing COS sequences from lambda phage), viral genomes comprising non-native nucleic acid sequences, and the like. When used in the context of describing a vector, the terms "donor" and 15 "acceptor" refer to the fact that one vector (the donor) will contain a nucleic acid sequence, referred to herein as the "transfer sequence," that is to be excised and transferred to another (the acceptor) vector. Any given vector can be a donor or an acceptor, depending on whether it is the vector from which a nucleic acid sequence is being transferred, or the vector into which a nucleic acid sequence is introduced.

20 Both donor and acceptor vectors contain site-specific recombination sequences, which are sequences of nucleic acids that are specifically recognized by a particular site-specific recombinase. Site specific recombinases, as the term is used herein, are enzymes that catalyze the excision and/or recombination of nucleic acid sequences, and may form intermediate complexes with the transfer sequence DNA 25 during the recombination event. These enzymes recognize a relatively short, unique nucleic acid sequence in the donor and acceptor vectors that serves as a site for both recognition and recombination. Recombinases particularly useful in the practice of the invention are those that function in a wide variety of cell types because such enzymes do not require any host specific factors and do not require ATP to function.

5 Examples of site-specific recombinases of this type include type I topoisomerases (S. Shuman, *J. Biological Chemistry* 266:11372-79, 1991), integrases (Argos, *et al.*, *EMBO J* 5:433-440, 1986), resolvases (Hallet and Sherratt, *FEMS Microbiol. Rev.* 21:157-178, 1997), and the like.

5 A particularly suitable enzyme for use in the practice of the invention is a type I topoisomerase, particularly vaccinia DNA topoisomerase. Vaccinia DNA
15 topoisomerase binds to duplex DNA and cleaves the phosphodiester backbone of one strand. The enzyme exhibits a high level of sequence specificity, akin to that of a restriction endonuclease. Cleavage preferentially occurs at a consensus
20 pentapyrimidine element 5'-(C/T)CCTT↓ (SEQ ID NO: 1) in the scissile strand. In the cleavage reaction, bond energy is conserved via the formation of a covalent adduct between the 3' phosphate of the incised strand and a tyrosyl residue of the
25 topoisomerase I protein. Vaccinia topoisomerase can religate the covalently held strand across the same bond originally cleaved (as occurs during DNA relaxation) or it
15 can ligate the strand to a heterologous acceptor DNA 5' end containing a site specific recombination site, such as the DNA in the invention acceptor vector, and thereby
30 create a new recombinant molecule, as shown in Figure 1C.

When the substrate is configured such that the scissile bond with the
topoisomerase is situated near (within about 10 to about 12 base pairs of) the 3' end of
35 a DNA duplex, cleavage is accompanied by the spontaneous dissociation of the downstream portion of the cleaved strand in the donor vector. The resulting
topoisomerase-DNA complex, containing a 5' single-stranded tail, can religate to an
40 acceptor DNA if the acceptor molecule has a 5' OH terminated acceptor strand with sequence (e.g. of at least a four base overhang) complementary to that of the activated
25 donor complex (i.e., the single strand tail of the noncleaved donor strand in the immediate vicinity of the scissile phosphate). In the absence of an acceptor strand, the
45 topoisomerase can transfer the CCTT strand to water, releasing a 3'-phosphate-terminated hydrolysis product, or to glycerol. However, the hydrolysis reaction is
50 much slower than religation to an acceptor DNA strand of the acceptor vector, the
30 extent of strand transfer to non-DNA nucleophiles being generally about 14-40%.

5 The specificity of vaccinia topoisomerase in DNA cleavage and its versatility
in strand transfer have inspired topoisomerase-based strategies for polynucleotide
synthesis in which DNA oligonucleotides containing CCCTT cleavage sites serve as
10 activated linkers for the joining of other DNA molecules with compatible termini (S.
Shuman, *J. Biol. Chem.* 269:32678-32684, 1994). The use of vaccinia topoisomerase
5 type I for cloning generally is described in detail in U.S. Patent No. 5,766,891, which
is incorporated by reference herein in its entirety.

Bivalent strand transfer also results in circularization of the acceptor vector
DNA by placing the topoisomerase cleavage sites on the transfer sequence (a
20 synthetic bivalent substrate) and cloning the cleaved DNA into the donor vector. This
strategy is well-suited to the cloning of DNA fragments amplified by PCR. To clone
PCR products using vaccinia topoisomerase, it is preferred to include a 10 nucleotide
sequence -5'-XXXXAAGGGC- (SEQ ID NO:2) at the 5' end of the two primers used
25 for amplification. The 5'-XXXX segment can correspond to any 4-base overhang that
is compatible with the restriction site into which the PCR product will ultimately be
15 cloned. The amplification procedure will generate duplex molecules containing the
sequence 5'-GCCCTTxxxx-3' (SEQ ID NO:3) at both 3' ends (where xxxx is the
complement of XXXX). Incubation of the PCR product with topoisomerase will
30 result in cleavage at both termini and allow the covalently activated PCR fragment to
be ligated into the donor vector DNA. From the donor vector the transfer sequence
35 can be simultaneously transferred to one or a number of different acceptor vectors
engineered to contain functional sequences suitable for accomplishing different types
of cloning procedures. For example, an acceptor vector that is a bacterial expression
40 vector generally includes a promoter (such as the lac promoter), the Shine-Dalgarno
sequence (for transcription initiation) and the start codon (AUG). Similarly, a
25 eukaryotic expression vector includes, but is not limited to, a heterologous or
homologous promoter for RNA polymerase II, a downstream polyadenylation signal,
45 the start codon AUG, and a termination codon for detachment of the ribosome.

The donor complex formed upon cleavage by topoisomerase at a 3' proximal
50 site is extremely stable. The transfer sequence can be transferred nearly quantitatively

5 to an acceptor vector with a complementary site even after many hours of incubation
of the covalent topo-DNA complex at room temperature. The topo-transfer sequence
complex can even be denatured with 6 M guanidine HCl and then renatured
10 spontaneously upon removal of guanidine with complete recovery of strand
transferase activity. Thus, a topoisomerase-activated vector can be prepared once in
5 quantity and used as many times as needed for preparation of various types of
acceptor vectors according to the invention.
15

In addition, two major families of site-specific recombinases from bacteria and
unicellular yeast have been described: the integrase family and the resolvase/invertase
20 family. In these recombinases, strand exchange catalyzed by site specific
recombinases occurs in two steps of (1) cleavage and (2) rejoining, involving a
covalent protein-DNA intermediate formed between the recombinase enzyme and the
DNA strand(s). The nature of the catalytic amino acid residue of the enzyme and the
25 line of entry of the nucleophile is different for these two recombinase families. For
cleavage catalyzed by the invertase/resolvase family, the nucleophile hydroxyl is
15 derived from a serine and the leaving group is the 3'-OH of the deoxyribose. For the
integrase family, the catalytic residue is a tyrosine and the leaving group is the 5'-OH.
30 In both recombinase families, the rejoining step is the reverse of the cleavage step.

The recombinase activity of Cre has been studied as a model system for the
35 integrases. Cre is a 38 kD protein isolated from bacteriophage P1. It catalyzes
recombination at a 34 base pair stretch of nucleic acids called loxP. The loxP site has
the sequence 5'-ATAACTTCGTATAGCATACATTATACGAAGTTAT-3' (SEQ ID
40 NO: 4; spacer region underlined), consisting of two 13 base pair palindromic repeats
flanking an eight basepair core sequence (Hoess *et al.*, *Proc. Natl. Acad. Sci USA*
25 79:3398, 1982 and U. S. Patent No. 4,959,217, the disclosure of which is herein
incorporated by reference in its entirety). The repeat sequences act as Cre binding
45 sites with the crossover point occurring in the internal spacer core. Each repeat
appears to bind one protein molecule wherein the DNA substrate (one strand) is
cleaved and a protein-DNA intermediate is formed having a 3'-phosphotyrosine
50 linkage between Cre and the cleaved DNA strand. Crystallography and other studies
55

5 suggest that four proteins and two loxP sites (each on a different DNA molecule) form
a synapsed structure in which the DNA resembles models of four-way Holliday-
10 junction intermediates, followed by the exchange of a second set of strands to resolve
the intermediate into recombinant products (see, Guo, *et al.*, *Nature* 382:40-46, 1997).

- 5 The asymmetry of the core region of the loxP recombination sequence is responsible
for directionality of the recombination reaction. When two loxP sites on the same
15 DNA molecule are in a directly repeated orientation, Cre excises the DNA between
these two sites, leaving a single loxP site on the DNA molecule (Abremski *et al.*, *Cell*
32:1301, 1983). Thus, the repeat sequences act as Cre-specific binding sites with the
10 recombination crossover point occurring in the core.

The loxP site is so complex in size that it occurs only in the P1 phage genome.
Therefore, use of the loxP sites in the invention vectors assures that the enzyme will
25 not cut the transfer sequence within the interior of the sequence unless the transfer
sequence is from the P1 phage genome. The activity of Cre in a wide variety of
15 cellular backgrounds, including yeast, shows that Cre does not require host specific
factors for activity (Sauer *Mol. Cell. Biol.* 7:2087-2096, 1987), plants (Albert *et al.*,
30 *Plant J.* 7:649-659, 1995; Dale and Ow, *Gene* 21:79-85, 1990; Odell *et al.*, *Mol. Gen.*
Genet. 223:369-378, 1990) and mammals, including both rodent and human cells (van
Deursen *et al.*, *Proc. Natl. Acad. Sci. USA* 92:7376-7380, 1995; Agah *et al.*, *J. Clin.*
20 *Invest.* 100:169-179, 1997; Sauer and Henderson, *New Biologist* 2:441-449, 1990).

The Cre protein also recognizes a number of variant or mutant lox sites
(variant relative to the loxP sequence), including the loxB, loxL and loxR sites, which
40 are found in the *E. coli* chromosome. Other variant lox sites include loxP511
(5'-ATAACTTCGTATAGTATACATTATACGAAGTTAT-3' (SEQ ID NO:5;
25 spacer region underlined); loxC2
(5'-ACAACCTTCGTATAATGTATGCTATACGAAGTTAT-3' (SEQ ID NO:6;
45 spacer region underlined; U.S. Patent No. 4,959,217). Additional variants of the loxP
site can be prepared by those of skill in the art and will generally have no more than a
total of one to three point mutations in the two repeats that comprise the site-specific
50 recombination sequence. Cre catalyzes the cleavage of the lox site within the spacer

5 region and creates a six base-pair staggered cut. The two 13 bp inverted repeat
domains of the lox site represent binding sites for the Cre protein. The two lox sites
may differ so long as Cre is able to recognize both lox sites. However, if two lox sites
10 differ in their spacer regions in such a manner that the overhanging ends of the
5 cleaved DNA cannot reanneal with one another, Cre cannot efficiently catalyze a
recombination event using the two different lox sites. The efficiency of the
15 recombination event will depend on the degree and the location of the variations in the
binding sites. For example, the loxC2 site can be efficiently recombined with the
loxP site because the two lox sites differ by a single nucleotide in the leftbinding site.
20 Thus, when Cre is the site specific recombinase used in the practice of the invention
methods, the site-specific recombination sequence is a loxP site, or a variant thereof
recognized by the Cre enzyme.

25 A recombinase of the integrase family with similar function is Flp, a
recombinase identified in strains of *Saccharomyces cerevisiae* that contain 2 μ -circle
15 DNA. Flp recognizes a DNA sequence consisting of two 13 basepair inverted repeats
flanking an 8 basepair core sequence
30 (5'-GAAGTTCCTATTCICTAGAAAGTATAGGAACTTC-3' (SEQ ID NO: 7);
spacer underlined) called *FRT* (Flp Recombination Target site). A third repeat
follows at the 3' end in the natural sequence, but does not appear to be required for
35 20 recombinase activity. The Flp gene has been cloned and expressed in *E. coli* and in
mammalian cells (PCT International Patent Application PCT/US92/01899,
Publication No: WO 92/15694, the disclosure of which is herein incorporated by
reference) and has been purified (Meyer-Lean *et al.*, *Nucleic Acids Res.* 15:6469,
40 1987; Babineau *et al.*, *J. Biol. Chem.* 260:12313, 1985; Gronostajski and Sadowski, *J.*
25 *Biol. Chem.* 260:12328, 1985).

45 Like Cre, Flp is functional in a wide variety of systems including bacteria
(Huang *et al.*, *J. Bacteriology* 179:6076-6083, 1997), insects (Golic and Lindquist,
Cell 59:499-509, 1989; Golic and Golic, *Genetics* 144:1693-1711, 1996), plants
(Lyznik *et al.*, *Nucleic Acids Res* 21:969-975, 1993) and mammals (U. S. Patent Nos.
50
55

5,677,177 and 5,654,182), which shows the Flp does not require host specific factors for operability.

Unlike the integrases, each member of the resolvase subfamily of recombinase enzymes contains an N-terminal catalytic domain having a high degree (>35%) of sequence homology among the subfamily members (Crellin and Rood, *J. Bacteriology* 179(16):5148-5156, 1997; Christiansen *et al.*, *J. Bacteriology* 178(17):5164-5173, 1996). Despite this, like the integrases, many of the resolvases do not require host specific accessory factors (Thorpe and Smith, *PNAS USA* 95:5505-5510, 1998).

Other site-specific recombinases suitable for use in the system and methods of the present invention include RecA (Ferrin *et al.*, *PNAS USA* 95:2156-57, 1998), HK022 integrase, lambda integrase (with or without Xis), which recognizes Att sites (Weisberg *et al.*, In: *Lambda II*, Hendrix *et al.*, Eds., Cold Spring Harbor Press, Cold Spring Harbor, NY, 1983), and the like.

The process of strand exchange used by the resolvases is somewhat different than the process used by the integrases. The resolvases usually make cuts close to the center of the crossover site, and the top and bottom strand cuts are often staggered by 2 basepairs, leaving recessed 5' ends. A protein-DNA linkage is formed between phosphodiester from the 5' DNA end and a conserved serine residue close to the amino terminus of the recombinase. Like the invertases, two proteins units are bound at each crossover site, however, no equivalent to the Holliday-junction intermediate is formed (see Stark *et al.*, *Trends in Genetics* 8(12):432-439, 1992, incorporated by reference herein).

The nucleic acid sequences recognized as recombination sites by members of the resolvase family differ in several ways from the integrases. The sites used for recognition and recombination of the phage and bacterial DNAs (the native host system) are generally non-identical, although they typically have a common core region of nucleic acids. The bacterial sequence is generally called the AttB sequence (bacterial attachment) and the phage sequence is called the AttP sequence (phage

5 attachment). Because AttB and AttP are somewhat different sequences,
recombination will result in a stretch of nucleic acids (called AttL or AttR for left and
right) that is neither an AttB sequence nor an AttP sequence, and is probably
10 unrecognizable as a recombination site to the relevant enzyme, thus reducing the
5 possibility that the enzyme will catalyze a second recombination reaction that would
reverse the first.

15 The individual resolvases and the nucleic acid sequences that they recognize
have been less well characterized than Cre and Flp, although most of the core
sequences have been identified. The core sequences of some of the resolvases useful
20 in the practice of the invention include TP901-1 - 5'-TTCAAT(T/C)AAGGTAA
(SEQ ID NO: 8); TnpX - 5'-GCCCNGA(G/A)GG (SEQ ID NO: 9), R4 - 5'-
GAAGCAGTGGTA (SEQ ID NO: 10), and ϕ C31 - 5'-TTG (SEQ ID NO: 11) (see
25 Rausch and Lehmann, *NAR* 19:5187-5189, 1991; Shirai *et al.*, *J. Bacteriology*
173(13):4237-4239, 1991; Crellin and Rood, *J Bacteriology* 179:5148-5156, 1997;
15 Christiansen *et al.*, *J Bacteriology* 176:1069-1076, 1994, all of which are incorporated
by reference herein.)

30 In general, Site-specific recombination sequences of the invention vary in
length, although they are generally less than 50 nucleotides. Particularly suitable site-
specific recombination sequences include the recognition sequences for vaccinia
35 topoisomerase I (5'-(C/T)CCTT↓, SEQ ID NO: 1), Cre (5'-ATAACTTCGTATA
GCATACAT TATACGAAGTTAT-, SEQ ID NO: 4), Flp (5'-GAAGTTCCTATAC
TTCTAGAA GAATAGGAAGTTC, SEQ ID NO: 7), lambda integrase
40 (5'-CAAGTT, SEQ ID NO: 12), HK022 integrase (5'-AACCTT, SEQ ID NO: 13),
and the like. The present invention is illustrated, but not limited by the use of vectors
25 containing topoisomerase I sites.

45 Any nucleic acid sequence is suitable as a transfer sequence as long as there is
a desire for the sequence to be moved from one vector to another. The transfer
sequence may, for example, encode a protein, peptide or functional RNA (such as
50 antisense sequences, hammerhead ribozymes, and the like). A transfer sequence

5 encoding a protein or peptide may be either a gene sequence or a coding sequence. As
used herein, a "gene sequence" is the entire nucleic acid sequence that is necessary for
the synthesis of a functional polypeptide or RNA molecule; whereas a "coding
10 sequence" is limited to the nucleic acids encoding the amino acid sequence of a
5 protein.

15 The transfer sequence may also be a sequence whose function, if any, is not
yet known, such as an expressed sequence tag (EST) fragment. Such sequences can
be used as diagnostic probes, or as aids in the identification and cloning of a larger
sequence containing the EST fragment.

20 10 The vectors employed in the practice of the invention contain one or more
nucleic acid sequences in addition to the site-specific recombination sequences, and
transfer sequence in the case of a donor vector. The additional nucleic acid sequences
25 will generally have some function in the replication or integrity of the vector, in the
expression of a protein, in the modification of an expressed protein, and the like.
15 Particularly useful nucleic acid sequences include promoter-enhancer sequences,
selection marker sequences, origins of replication, inducible element sequences,
30 fusion protein producing sequences, for example, localization signal sequences,
epitope tags, proteolytic cleavage recognition sequences, polypeptides that facilitate
purification, and the like.

35 20 Promoter-enhancer sequences are DNA sequences to which RNA polymerase
binds and initiates transcription. The promoter determines the polarity of the
transcript by specifying which strand will be transcribed. Bacterial promoters consist
40 of consensus sequences, -35 and -10 nucleotides relative to the transcriptional start,
which are bound by a specific sigma factor and RNA polymerase. Eukaryotic
25 promoters are more complex. Most promoters utilized in vectors are transcribed by
RNA polymerase II. General transcription factors (GTFs) first bind specific
45 sequences near the start and then recruit the binding of RNA polymerase II. In
addition to these minimal promoter elements, small sequence elements are recognized
50 specifically by modular DNA-binding/trans-activating proteins (e.g. AP-1, SP-1) that

5 regulate the activity of a given promoter. Viral promoters serve the same function as
bacterial or eukaryotic promoters and either provide a specific RNA polymerase in
trans (bacteriophage T7) or recruit cellular factors and RNA polymerase (SV40, RSV,
10 CMV). Viral promoters may be preferred as they are generally particularly strong
5 promoters.

15 Promoters may be, furthermore, either constitutive or regulatable (i.e.,
inducible or derepressible). Inducible elements are DNA sequence elements which
act in conjunction with promoters and bind either repressors (e.g. lacO/LAC Iq
repressor system in *E. coli*) or inducers (e.g. gal1/GAL4 inducer system in yeast). In
20 either case, transcription is virtually "shut off" until the promoter is derepressed or
10 induced, at which point transcription is "turned-on".

25 Examples of constitutive promoters include the int promoter of bacteriophage
 λ , the bla promoter of the β -lactamase gene sequence of pBR322, the CAT promoter
of the chloramphenicol acetyl transferase gene sequence of pPR325, and the like.
15 Examples of inducible prokaryotic promoters include the major right and left
promoters of bacteriophage (P_L and P_R), the trp, reca, lacZ, LacI, AraC and gal
30 promoters of *E. coli*, the α -amylase (Ulmanen *et al.*, *J. Bacteriol.* 162:176-182, 1985)
and the sigma-28-specific promoters of *B. subtilis* (Gilman *et al.*, *Gene Sequence*
32:11-20, 1984), the promoters of the bacteriophages of *Bacillus* (Gryczan, In: *The*
35 *Molecular Biology of the Bacilli*, Academic Press, Inc., NY, 1982), *Streptomyces*
20 promoters (Ward *et al.*, *Mol. Gen. Genet.* 203:468-478, 1986), *Pichia* promoters
(U.S. Patent Nos. 4,855,231 and 4,808,537), and the like. Exemplary prokaryotic
40 promoters are reviewed by Glick (*J. Ind. Microbiol.* 1:277-282, 1987); Cenatiempo
(*Biochimie* 68:505-516, 1986); and Gottesman (*Ann. Rev. Genet.* 18:415-442, 1984).

25 Preferred eukaryotic promoters include, for example, the promoter of the
mouse metallothionein I gene sequence (Harner *et al.*, *J. Mol. Appl. Gen.* 1:273-288,
45 1982); the TK promoter of Herpes virus (McKnight, *Cell* 31:355-365, 1982); the
SV40 early promoter (Benoist *et al.*, *Nature (London)* 290:304-310, 1981); the yeast
50 *gal1* gene sequence promoter (Johnston *et al.*, *Proc. Natl. Acad. Sci. (USA)* 79:6971-

5 6975, 1982); Silver *et al.*, *Proc. Natl. Acad. Sci. (USA)* 81:5951-5955, 1984), the
CMV promoter, the EF-1 promoter, Ecdysone-responsive promoter(s), tetracycline-
10 responsive promoter, and the like.

Selection marker sequences are valuable elements in expression vectors as
5 they provide a means to select for growth only those cells which have been
successfully transformed with a vector containing the selection marker sequence and
15 express the marker. Such markers are of two types: drug resistance and auxotrophic.
A drug resistance marker enables cells to detoxify an exogenously added drug that
would otherwise kill the cell. Auxotrophic markers allow cells to synthesize an
20 10 essential component (usually an amino acid) while grown in media which lacks that
essential component.

Common selectable marker gene sequences include those for resistance to
25 antibiotics such as ampicillin, tetracycline, kanamycin, bleomycin, streptomycin,
hygromycin, neomycin, Zeocin™, and the like. Selectable auxotrophic gene
15 sequences include, for example, hisD, which allows growth in histidine free media in
the presence of histidinol.

A further element useful in a vector is an origin of replication sequence.
Replication origins are unique DNA segments that contain multiple short repeated
35 sequences that are recognized by multimeric origin-binding proteins and which play a
20 key role in assembling DNA replication enzymes at the origin site. Suitable origins of
replication for use in expression vectors employed herein include *E. coli oriC*, colE1
plasmid origin, 2 μ and ARS (both useful in yeast systems), sfl, SV40 EBV oriP
40 (useful in mammalian systems), and the like.

Fusion protein producing sequences may be included in a vector employed in
25 the present invention. When two protein-coding sequences not normally associated
45 with each other in nature are in the same reading frame the resulting expressed protein
is called a "fusion protein" as two distinct proteins and/or fragments have been
"fused" together. Fusion proteins have a wide variety of uses. For example, two
50 functional enzymes can be fused to produce a single protein with multiple enzymatic

5 activities or short peptide sequences can be fused to a larger protein and serve as aids
in purification or as means of identifying expressed protein by serving as epitopes
detectable by specific antibodies. Thus examples of fusion protein producing
10 sequences useful in the vectors of the invention include epitope-tag encoding
5 sequences, affinity purification-tag encoding sequences, functional protein encoding
sequences, and the like.

15 Epitope tags are short peptide sequences that are recognized by epitope
specific antibodies. A fusion protein comprising a recombinant protein and an epitope
tag can be simply and easily purified using an antibody bound to a chromatography
20 10 resin. The presence of the epitope tag furthermore allows the recombinant protein to
be detected in subsequent assays, such as Western blots, without having to produce an
antibody specific for the recombinant protein itself. Examples of commonly used
epitope tags include V5, glutathione-S-transferase (GST), hemagglutinin (HA), the
25 peptide Phe-His-His-Thr-Thr, chitin binding domain, and the like.

15 Affinity purification tags are generally peptide sequences that can interact with
a binding partner immobilized on a solid support. Preferably, the recombination event
30 in the invention method places the transfer sequence in frame with the sequence
encoding the affinity domain, so that the affinity purification tag and the expression
product of the transfer sequence is expressed as a fusion protein when the sequence is
35 20 expressed. DNA sequences encoding multiple consecutive single amino acids, such
as histidine, when fused to the expressed protein, may be used for one-step
purification of the recombinant protein by high affinity binding to a resin column,
such as nickel sepharose. An endopeptidase recognition sequence can be engineered
40 between the polyamino acid tag and the protein of interest to allow subsequent
25 removal of the leader peptide by digestion with enterokinase, and other proteases.
Sequences encoding peptides, such as the chitin binding domain (which binds to
chitin), glutathione-S-transferase (which binds to glutathione), biotin (which binds to
45 avidin and streptavidin), and the like, can also be used for facilitating purification of
the protein of interest. The affinity purification tag can be separated from the protein
50

of interest by methods well known in the art, including the use of inteins (protein self-splicing elements, Chong *et al.*, *Gene* 192:271-281, 1997).

The use of the term "functional protein encoding sequence", as used herein, indicates that the fusion protein producing element of a vector encodes a protein or peptide having a particular activity, such as an enzymatic activity, a binding activity, and the like. For example, a functional protein encoding sequence may encode a kinase catalytic domain (Hanks and Hunter, *FASEB J* 9:576-595, 1995), producing a fusion protein that can enzymatically add phosphate moieties to particular amino acids, or may encode a Src Homology 2 (SH2) domain (Sadowski, *et al.*, *Mol. Cell. Bio.* 6:4396, 1986; Mayer and Baltimore, *Trends Cell. Biol.* 3:8, 1993), producing a fusion protein that specifically binds to phosphorylated tyrosines.

The foregoing elements can be combined to produce vectors suitable for use in the methods of the invention. Those of skill in the art would be able to select and combine the elements suitable for use in any particular system.

Suitable prokaryotic vectors include plasmids such as those capable of replication in *E. coli* (for example, pBR322, ColEI, pSC101, PACYC 184, itvX, pRSET, pBAD (Invitrogen, Carlsbad, CA), and the like). Such plasmids are disclosed by Sambrook (cf. *Molecular Cloning: A Laboratory Manual*, second edition, edited by Sambrook, Fritsch, & Maniatis, Cold Spring Harbor Laboratory, 1989). *Bacillus* plasmids include pCI94, pC221, pTI27, and the like, and are disclosed by Gryczan (In: *The Molecular Biology of the Bacilli*, *supra*, pp. 307-329). Suitable *Streptomyces* plasmids include pIJ101 (Kendall *et al.*, *J. Bacteriol.* 169:4177-4183, 1987), and streptomyces bacteriophages such as ϕ C31 (Chater *et al.*, In: *Sixth International Symposium on Actinomycetales Biology*, Akademiai Kiado, Budapest, Hungary, pp. 45-54, 1986). *Pseudomonas* plasmids are reviewed by John *et al.* (*Rev. Infect. Dis.* 8:693-704, 1986), and Izaki (*Jpn. J. Bacteriol.* 33:729-742, 1978).

Suitable eukaryotic plasmids include, for example, BPV, EBV, vaccinia, SV40, 2-micron circle, pcDNA3.1, pcDNA3.1/GS, pYES2/GS, pMT, pIND, pIND(Sp1), pVgRXR (Invitrogen), and the like, or their derivatives. Such plasmids

are well known in the art (Botstein *et al.*, *Miami Wntr. Symp.* 19:265-274, 1982; Broach, In: *The Molecular Biology of the Yeast Saccharomyces: Life Cycle and Inheritance*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY pp. 445-470, 1981; Broach, *Cell* 28:203-204, 1982; Dilon *et al.*, *J. Clin. Hematol. Oncol.* 10:39-48, 1980; Maniatis, In: *Cell Biology: A Comprehensive Treatise*, Vol. 3, Gene Sequence Expression, Academic Press, NY, pp. 563-608, 1980.

A further embodiment of the invention comprises a method of rapidly subcloning a nucleic acid sequence. The invention method comprises contacting a site-specific recombinase and a cell-free solution comprising a donor vector comprising a transfer sequence flanked by a site-specific recombination sequence recognized by the recombinase, and an acceptor vector comprising at least one site-specific recombination sequence recognized by the recombinase, under conditions suitable to promote the transfer of the transfer sequence from the donor vector to the acceptor vector. The invention method employs vectors and recombinases as described above. Means of identifying conditions for the transfer of a transfer sequence from a donor vector to an acceptor vector can readily be determined by those of skill in the art. Suitable conditions include those described in Nunes-Düby *et al.*, *EMBO J.* 13(18):4421-4430, 1994; Senecoff *et al.*, *PNAS USA* 82:7270-7274, 1985; Shaikh and Sadowski, *J. Biol. Chem.* 272(9):5695-5702, 1997; and Peterson and Shuman, *J. Biol. Chem.* 272(7):3891-3896, 1997, all of which are incorporated by reference herein, and are described in detail in the Examples set out below.

For example, the invention method can be used to perform subcloning (transfer of a DNA or RNA sequence from one vector to another) without PCR amplification using topoisomerase, as described in Examples 1A-C below. In this embodiment of the invention, donor vector is constructed as shown in Figure 1 with recognition sites for vaccinia topoisomerase I flanking the insertion point for the transfer sequence. The two recognition sites are juxtaposed on opposite strands of the DNA and are generally separated by about four spacer nucleotides to provide overhang. The spacer nucleotides have identical sequences on either side of the insertion point for the gene of interest. One or more vectors is prepared as a linear,

5 double-stranded molecule with single strand overhangs that are compatible with the
spacer sequences that flank the gene or gene fragment of interest on the donor vector,
as shown in Figure 1B. In addition, the linear acceptor vector DNA has 5'-hydroxyl
10 groups at each end. A marker gene sequence and additional sequences are included in
the acceptor vector as known in the art depending upon the particular attribute of the
vector desired. Multiple acceptor vectors useful for different cloning tasks can be
15 simultaneously prepared by including in each those attributes suitable to the task for
which the vector would be used.

The donor vector(s) are treated with topoisomerase I for five minutes at room
20 temperature. The enzyme generates nicks at each topoisomerase recognition site,
creating double strand breaks at the sites that flank the inserted gene or gene fragment
of interest and releasing the transfer DNA fragment. Topoisomerase I is covalently
25 attached at each end of the freed DNA fragment, which also has overhangs
complementary to the spacer nucleotides. The topoisomerase treated vector is
15 combined with the linearized acceptor vector in a suitable medium. The compatible
ends of each vector corresponding to the spacer sequence brings the two DNA
fragments together and allows the topoisomerase I to ligate the spacer sequences
30 together in an ATP independent ligation. The recombinant vector formed, shown in
Figure 1C, contains the gene or gene fragment of interest and can be identified
20 following transformation of the vector into competent E. coli by expression of the
marker gene. When Cre or Flp is used as the site specific recombinase, the donor and
acceptor vectors are prepared as described in Example 1 except that the recognition
sites appropriate to the recombinase of choice flank the insertion point for the gene of
40 interest.

25 In another embodiment of the invention method, a gene or gene fragment
donor clones are created by PCR amplification cloning using primers designed for the
specific fragment of interest. A donor vector is not needed. The gene or gene
45 fragment of interest is generated repeatedly from the donor clone for insertion into any
or all of the acceptor vectors for a wide variety of research or production applications.
50 No subcloning is required in this technique to move the gene of interest from one

5 vector into another. The gene or gene fragment is simply copied from a donor clone, and the copies are inserted into a "copy ready vector" using the following procedure.

10 In this procedure, the exact sequence of the open reading frame, if any, and of native features of the gene to be transferred should be noted if the gene is to be
5 expressed as a fusion protein from one or more of the acceptor vectors. For example, signal sequences for intracellular organelle targeting, secretion, glycosylation, etc. are
15 identified in the transfer sequence to determine that the gene of interest is in reading frame with any signal sequence or genes encoding a tag, and the like, in the acceptor vector.

20 10 Oligonucleotides are designed for PCR amplification of the exact DNA sequence to be transferred to the acceptor vector(s) using one or more methods well known in the art. For example, to transfer a complete open reading frame, the
25 sequence of one oligonucleotide would have the translation initiation codon at its 5'-end and the sequence of the other oligonucleotide would have the translation initiation
15 codon at its 5'-end. The sequence of the other oligonucleotide would have the complement of the translation termination codon at its 3'-end. Acceptor vectors are prepared as described in Example 1, such as an acceptor vector including DNA
30 sequences appropriate for the expression or analysis of the protein encoded by the gene of interest.

35 20 The gene sequence of interest is amplified from the donor clone using the PCR primers prepared as above-described, with cycling parameters selected as suitable for the primer and the template. A 7 to 30 minute extension at 72°C is optionally
40 included to ensure that all amplified products are full length and 3' adenylated. The amplified DNA fragment is ligated into the acceptor vector(s). In general, 0.5 to 2 µl
25 of the PCR product (10 ng/µl) with an average insert length of 400 to 100 bp gives a proper insert:vector ratio. Therefore the PCR product is ligated into the acceptor
45 vector by placing 0.5 to 2 µl of PCR product reaction in sterile water to provide a final volume of 4 µl. To this mixture is added 1 µl of the acceptor vector to obtain a final
50 volume of 5 µl., mixing gently and incubating for 5 minutes at room temperature

5 (25° C), then centrifuging briefly and placing the tube on ice. Competent cells, such as *E. coli*, are then immediately transformed with the acceptor vector(s).

10 In yet another embodiment of the invention method, gene or gene fragment clones are created by PCR amplification using primers designed specifically, or non-specifically, for the fragment, but which also contain sequences that, when the
15 amplified gene fragment is inserted into an invention donor vector, will allow use of a universal donor vector primer set to create copies of the gene or gene fragment for insertion into one or more specialty application acceptor vectors using the following
20 procedure. If a collection of genes are to be transferred, each gene of interest should be available on a donor plasmid vector and flanked by short sequences that are common to all donor plasmids in the collection. Oligonucleotides for PCR
25 amplification of the gene(s) are synthesized based on the short sequence that flanks each of the transfer sequences in the donor vectors.

An invention acceptor vector containing a recombinase recognition site
15 appropriate for the expression or analysis of the gene of interest is selected. For example, the acceptor vector containing a topoisomerase I recognition site, a strong mammalian promoter, and the coding sequence for an epitope tag would be
30 appropriate for production and analysis of the protein of interest, such as the TOPO Cloning™ vector (Invitrogen, Carlsbad, CA). The transfer sequence(s) of interest are
35 amplified from the donor vector using the PCR primers with cycling parameters suitable for the particular primers and template. It may be necessary to include a 7 to
40 30 minute extension of 72°C to ensure that all amplified products are full length and 3' adenylated. The amplified DNA fragments are individually transferred into
acceptor vectors) using the insert:vector ratio and conditions described above.

25 In a presently preferred embodiment of the invention method, the PCR primers
45 add the following sequences at the 5' end to add topoisomerase I recognition sites to the ends of the amplified PCR product:

Forward Primer 5'-AAGGG (SEQ ID NO:14)

Reverse Primer 5'-CCCTT (SEQ ID NO:1)

The acceptor vector is prepared as a linear molecule with single 3'-T overhangs and 5'-hydroxyl groups. After amplification by PCR, the PCR product is treated with topoisomerase I so that the enzyme becomes covalently bound to each end of the amplified PCR product. Then the covalently bound PCR product is introduced into the acceptor vector(s) as described above.

In another embodiment, the invention provides kits comprising one or more containers or vials containing components for carrying out the methods of the present invention. For instance, such a kit can comprise a suitable reaction solution, recombinase and cells. Also included in the kit are one or more vectors, e.g., vectors for expression in mammalian, bacterial, yeast and insect cells. In a preferred embodiment, the kit will comprise a reaction solution of 50 mM Tris HCl pH 7.5, one or more of the invention vectors that have vaccinia DNA topoisomerase covalently bound thereto, and instructions for their use as described herein.

In one embodiment the invention kit comprises at least one donor vector comprising at least one site specific recombination sequence, a transfer sequence, and a first selectable marker, and at least one acceptor vector comprising at least one site specific recombination sequence, a lethal gene and a second selectable marker. For example, as illustrated in Figure 2, the donor vector in the kit can contain a selectable marker gene other than Zeocin, an origin of replication sequence ("ori"), and a transfer sequence ("gene of interest") flanked by lox P recognition sites. The acceptor vector ("pAcceptor") then contains a gene encoding resistance to the antibiotic Zeocin™ ("Zeo"), an origin of replication sequence ("ori"), and a gene encoding ccdB, a lethal compound, flanked by loxP sites. When the donor and acceptor vectors are combined in a reaction mixture in the presence of the recombinase Cre, a new recombinant vector ("pRecombinant") is created, which recombinant vector contains the transfer sequence and a gene encoding Zeo. Cells transformed with the reaction mixture will grow in the presence of the antibiotic Zeocin™ only if the recombination event has successfully occurred.

Vaccinia DNA topoisomerase can be prepared for expression in *E. coli* and purified as described in S. Shuman *et al.*, *J. Biol. Chem.* **263**:16401-16407, 1988.

The invention will now be described in greater detail by reference to the following non-limiting Examples.

EXAMPLE 1

Subcloning without PCR Amplification using topoisomerase

Donor vectors can be constructed such that recognition sites for topoisomerase, or other ATP independent enzymes, flank the transfer sequence. In the presence of acceptor vector and topoisomerase, or other ATP independent enzyme, the transfer sequence is occasionally subcloned from the donor vector to the acceptor vector in an ATP independent event.

A linear activated vector containing vaccinia topoisomerase recognition sites (e.g., pCR2.1-TOPO (Invitrogen)) is prepared to receive the transfer sequence. The transfer sequence is amplified from a DNA template of choice. The DNA template may be genomic DNA, plasmid DNA, cosmid DNA or any other shuttle construct. Isolation methods are available in the public domain (Ausubel *et al.*, Section 2.14). Specific oligos (primers) for PCR corresponding to the exact sequence of the transfer DNA are synthesized according to published protocols (Ausubel *et al.*, Section 2.11). Both primers contain 7-9 additional bases on the 5' ends including the complement to the vaccinia topoisomerase I recognition site (5'-AAGGG 3') and an additional 2-4 bases which will serve as the 5' overhangs during subcloning with topoisomerase (5'-CGAAGGG . . . 3', SEQ ID NO:15). PCR amplification is performed utilizing methods optimized for the template and primers (Ausubel *et al.*, Section 15.1) with a DNA polymerase containing terminal transferase activity, such as Taq (Boehringer Mannheim, Indianapolis, IN).

Approximately 20ng of PCR product is combined with 1µl of the prepared activated vector in a total volume of 5µl. The reaction is incubated at 25°C for 5 min, placed on ice and 1µl is transformed into competent *E. coli* using either chemical transformation or electroporation techniques (Ausubel *et al.*, Section 1.8). Transformed cells are plated on appropriate antibiotic selection plates and grown at 37°C for 12-18 hours. Resulting colonies are screened by miniprep and restriction digest (Ausubel *et al.*, Sections 1.6 and 3.1) to identify clones containing transfer sequence.

Positive clones will contain the transfer sequence flanked on each side by 2 tandem topoisomerase recognition sites on complementary strands separated by 2-4 bases (for example, a direct repeat of 5'-CCCTTGCAAGGG (SEQ ID NO:16) with an intervening transfer sequence). A positive clone is propagated in *E. coli* and the plasmid DNA is purified as described above. The plasmid DNA is resuspended in TE Buffer, pH 8 (10mM Tris, 1mM EDTA) at a concentration of 10 ng/µl. This vector will serve as the donor for subcloning in an ATP independent reaction using topoisomerase.

B. Preparation of the Acceptor Vector

Preparation of linear, dephosphorylated vector: Supercoiled plasmid DNA to be used for construction of the acceptor vector is propagated and purified as described above. The plasmid chosen to be the acceptor vector must have a different *E. coli* antibiotic selection marker from the donor vector, for example Zeocin™. Plasmid DNA is digested with a restriction enzyme that is unique within the vector and will leave the desired 2-4 base 5' overhangs (e.g., digestion with *BstB I* will leave 2 base 5' overhangs). It is possible to digest with two different enzymes for directional cloning, however the forward and reverse PCR primers used to create the donor vector must be designed to generate the necessary complementary overhangs.

Plasmid DNA (30µg) is digested with 120 units of *BstB I* (New England BioLabs, Beverly, MA) for 2 hours under conditions specified by the supplier, extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1), ethanol precipitated, and washed with 500µl of 80% ethanol (Ausubel *et al.*, Section

5 2.1). The DNA ends are dephosphorylated by treating with calf intestinal alkaline
phosphatase (CIP; New England BioLabs, Beverly, MA) according to protocol
specified by the supplier, extracted with phenol/chloroform/isoamyl alcohol (25:24:1),
10 ethanol precipitated, and washed with 80% ethanol (Ausubel *et al.* Section 2.1). The
5 DNA is resuspended in 1000µl of TE buffer, pH 8.

15 C. Subcloning with Topoisomerase

Cell-free subcloning and selection: 10ng of prepared donor vector, 30ng of
prepared acceptor vector and 1µg of purified topoisomerase are combined in a total
20 volume of 5µl, and incubated for 5 min. at 25°C to allow transfer of the desired
10 sequence from the donor vector to the acceptor vector in an ATP independent
reaction. The reaction mixture is placed on ice and 1µl is transformed into competent
E. coli using either chemical transformation or electroporation techniques (Ausubel *et*
25 *al.*, Section 1.8). Clones containing acceptor vector plus transfer sequence are
selected by plating on antibiotic media requiring a resistance marker specific to the
15 acceptor vector (e.g., Zeocin™). Plates are incubated at 37°C for 12-18 hours.
Resulting colonies are screened by miniprep and restriction digest (Ausubel *et al.*,
30 Sections 1.6 and 3.1) to identify clones containing the desired transfer sequence
subcloned into the acceptor vector.

35 EXAMPLE 2

20 Protocol 2: Subcloning without PCR Amplification Using Site-Specific
Recombinases

40 A. Preparation of Donor Vector

Construct Design: A donor vector is constructed so that a transfer sequence
45 and a unique bacterial selection marker (e.g. Zeocin™, Invitrogen Corp., Carlsbad,
25 CA) are flanked by tandemly repeated recombinase recognition sites (for example
loxP or *FRT*). The donor vector construct containing recombinase recognition sites is
built using standard molecular biology techniques of PCR and subcloning (Ausubel *et*
50 *al.*

5 *al.*, Sections 3.16 and 3.17). The desired transfer sequence may be subcloned into the
donor vector using either standard PCR/restriction digest and ligation techniques
10 (Ausubel *et al.*, Sections 3.16 and 3.17) or by topoisomerase mediated cloning of PCR
products as described in Examples 3 and 5 hereafter.

5 **Donor Vector Preparation:** The donor plasmid DNA is propagated in *E. coli*
(see Example 1: Section A) and purified from 100 ml of a saturated culture according
15 to protocols specified for the SNAPTM Midiprep Kit (Invitrogen, Carlsbad, CA). The
plasmid DNA is resuspended in TE Buffer, pH 8 (10mM Tris, 1mM EDTA) at a
concentration of 0.5µg/µl.

20 10

25 **B. Preparation of the Acceptor Vector**

Construct Design: The acceptor vector contains a single recombination
recognition site in the desired cloning region that is identical to the two sites on the
30 donor vector. It also contains a bacterial selection marker that differs from that of the
15 donor vector (e.g., Ampicillin) to allow for selection of acceptor vector clones. The
acceptor vector is built using standard molecular biology techniques of PCR and
subcloning (Ausubel *et al.*, Sections 3.16 and 3.17).

Acceptor Vector Preparation: The acceptor plasmid DNA is propagated in
20 *E. coli* (Example 1: Section A above) and purified from 100ml of a saturated culture
according to protocols specified for the SNAPTM Midiprep Kit (Invitrogen, Carlsbad,
40 CA). The plasmid DNA is resuspended in TE Buffer pH 8 at a concentration of
0.5µg/µl.

45 **C. Subcloning with a Site-Specific Recombinase**

25 **Recombinase (Cre) Reaction:** A combination of 0.25µg of donor vector,
0.75µg of acceptor vector, 6µl of 10X Cre Buffer (50mM Tris-HCl, pH 7.5, 33mM
50

5 NaCl, 10mM MgCl₂, 100µg/ml BSA) and 2 units of Cre Recombinase (Novagen,
Madison, WI) is prepared in a 60µl total volume and incubated at 37°C for 15 min.
10 Competent *E. coli* are transformed with 2µl of the combination using either chemical
transformation or electroporation techniques (Ausubel *et al.*, Section 1.8). Based on
5 incompatibility of different vectors containing the same origin of replication within a
single cell (*Molecular Cloning, A Laboratory Manual*, Second Edition, Ed. Sambrook
15 *et al.*, Cold Spring Harbor Laboratory Press, New York, 1989, p. 1.4), clones
containing acceptor vector plus transfer sequence are selected by plating on antibiotic
media requiring resistance markers specific to both the acceptor vector and the donor
10 vector region that is subcloned (e.g., Ampicillin and Zeocin™). Plates are incubated
at 37°C for 12-18 hours. The resulting colonies are screened by miniprep and
restriction digest (Ausubel *et al.*, Sections 1.6 and 3.1) to identify clones containing
the desired transfer sequences and subcloned into the acceptor vector.

25 EXAMPLE 3

15 Cloning PCR amplified DNA with gene specific primers and Cloning vector.

30 Gene or gene fragment amplimers are created by PCR amplification using
primers sequence-specific to the gene or gene of interest. Any region of DNA
containing the gene of interest (designated the donor) and primers specific to the gene
35 of interest can be used to generate the amplimer repeatedly for insertion into any or all
20 of the acceptor vectors for a wide variety of research or production applications. No
subcloning is required in this technique to transfer the gene or gene fragment of
interest into the acceptor vector. The amplimer is simply copied off from the donor
40 and the copies inserted into the acceptor vector using the procedure described below.

5 The DNA template should be available in sufficient quantities (at least 20 ng
for plasmids) and the complete sequence of the target open reading frame should be
known. DNA template may be genomic DNA, plasmid DNA, cosmid DNA or any
10 other shuttle construct. Isolation methods are those known in the art, for example, as
disclosed in Ausubel *et al.*, Section 2.14.

Specific oligonucleotides (primers) for PCR corresponding to the exact DNA
15 sequence to be transferred to the acceptor vector are prepared. For example, to
transfer a complete open reading frame, the sequence of the 5' primer would contain
the translation initiation codon and flanking sequences of the target sequence. The
20 sequence of the 3' primer would contain the complement of the translation
termination sequence of the target. Protocols describing the synthesis of
oligonucleotides are available in the public domain (Ausubel *et al.*, Section 2.11).

25 An acceptor vector appropriate for the expression or analysis of the gene or gene
fragment of interest is TOPO Cloning™ vector, having the topoisomerase already
associated with the linear plasmid, for example, pCR2.1TOPO™ (Invitrogen,

30 The transfer sequence of interest is obtained from the donor clone in a 50 µl
reaction volume using the PCR primers specific to the transfer sequence. Cycling
parameters are selected to be appropriate for the primers and template used (Ausubel
35 *et al.*, Section 15.1). It may be necessary to include a 7 to 30 minute extension at
72°C after PCR is complete to ensure that all amplicons are full length and 3'
20 adenylated (Ausubel *et al.*, Section 15.7).

40 The amplicon is cloned into the acceptor vector as follows: For one
reaction, 0.5 to 2 µl fresh PCR product is combined with 1 µl of the acceptor
vector and sterile water is added to a 5 µl total volume. The mixture is gently
25 stirred and incubated for 5 minutes at room temperature (~25°C) and then
competent *E. coli* cells are immediately transformed with the mixture by any
45 known method.

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In general, 0.5 to 2 μ l of a typical PCR reaction (10 ng/ μ l) with an average amplicon length of 400 to 1000 bp will give the proper insert:vector ratio.

EXAMPLE 4

Cloning PCR amplified DNA with generic primers and a cloning vector.

Gene or gene fragment amplicons are created by PCR amplification using primers of sequence specific to the donor vector and unrelated to the transfer sequence (generic). Any plasmid containing the transfer sequence (designated the donor plasmid) and primers specific to the donor plasmid can be used to generate the amplicon repeatedly for insertion into any or all of the acceptor vectors for a wide variety of research or production applications. No subcloning is required in this technique to transfer the gene or gene fragment of interest into the acceptor vector. The amplicon is simply copied off from the donor plasmid and the copies inserted into the acceptor vector using the procedure described below.

The donor plasmid should be available in sufficient quantities (at least 20 ng) and the complete sequence of the target open reading frame should be known. Isolation methods are well known in the art (Ausubel *et al.*, Section 2.14).

Specific oligonucleotides (primers) are prepared corresponding to the plasmid DNA sequences flanking the amplicon to be transferred to the acceptor vector. Primers need to be made corresponding to regions of the plasmid immediately upstream and downstream of the amplicon. Protocols describing the synthesis of oligonucleotides are well known in the art (Ausubel *et al.*, Section 2.11).

An acceptor vector appropriate for the expression or analysis of the gene or gene fragment of interest and having the topoisomerase already associated with the linear plasmid is prepared. Such vectors are commercially available as TOPO Cloning™ vector, having the topoisomerase already associated with the linear plasmid, for example, pCR2.1 TOPO™ (Invitrogen, Carlsbad, CA).

5 The transfer sequence is cloned from the donor plasmid in a 50 µl reaction
volume using the PCR primers specific to the donor plasmid, and utilizing cycling
10 parameters that are appropriate for the primers and template as described in Example
3 above. The amplimer is cloned into the acceptor vector as described in Examples 1-
5 3 above.

15 EXAMPLE 5

Transferring PCR amplified DNA treated with topoisomerase.

20 A desired transfer sequence is amplified from a donor clone by PCR using
10 primers specific for the transfer sequence. The inclusion of topoisomerase recognition
sites at the 5' ends of the PCR primers enables transfer of the amplified sequence to
25 an appropriate acceptor vector when treated with topoisomerase.

A. PCR Amplified Transfer DNA

30 Preparation of amplified transfer DNA treated with topoisomerase: A
15 donor clone may be genomic DNA, cDNA, plasmid DNA, cosmid DNA or any other
shuttle construct. DNA from the donor clone is prepared for use as a template in PCR
amplification utilizing an appropriate preparation technique (Ausubel et. al., Sections
35 2.11 and 5.5). The sequence of the transfer DNA is known. DNA PCR primers
containing the complement of the vaccinia topoisomerase I recognition site (SEQ ID
20 NO:14 followed by transfer DNA specific sequence are synthesized according to
known protocols (Ausubel et. al, Section 2.11). The DNA fragment generated using
40 these primers will contain topoisomerase recognition sites at the 3' ends. An
additional 2-4 bases may be added at the 5' ends of each primer to create 5' overhangs
45 in the amplified DNA after treatment with topoisomerase. For example, including
25 SEQ ID NO:14 in the primer will result in 5' overhangs complementary to those
generated by digestion with *EcoR I*.

5 The transfer sequence is amplified by PCR following established methods
(Ausubel et. al, Section 15.1). 200ng of amplification product, 200ng of purified
10 topoisomerase I and TE buffer, pH 8 (10mM Tris, 1mM EDTA) are combined in a
total volume of 20 μ l. The reaction is incubated at 25°C for 5 min and placed on ice.
5 The topoisomerase will be covalently bound to the 3' ends of the PCR product,
leaving the desired 5' overhangs.

15 **B. Preparation of the Acceptor Vector**

Preparation of linear, dephosphorylated vector: Supercoiled plasmid DNA
to be used for construction of the acceptor vector is propagated and purified as
20 described previously (Example 1, Section A). The plasmid chosen to be the acceptor
vector should have a different *E. coli* antibiotic selection marker from the donor
vector. Plasmid DNA is digested with a restriction enzyme that is unique within the
25 vector and will leave the desired 2-4 base 5' overhangs (e. g. digestion with *EcoR I*
will leave 4 base 5' overhangs: 5'-AATT...-3'). It is possible to digest the acceptor
15 vector with two different enzymes for directional cloning, however the forward and
reverse PCR primers used to create the amplified transfer DNA must be designed to
30 generate the necessary complementary overhangs.

The supercoiled DNA of the acceptor vector is digested with 120 units of
35 *EcoR I* (New England BioLabs, Beverly, MA) for 3 hours under conditions specified
20 by the supplier, extracted with an equal volume of phenol/chloroform/isoamyl alcohol
(25:24:1), ethanol precipitated, and washed with 500 μ l of 80% ethanol (Ausubel et.
al, Section 2.1). Ends of the DNA are dephosphorylated by treating with calf
40 intestinal alkaline phosphatase (CIP; New England BioLabs, Beverly, MA) according
to protocol specified by the supplier, then the DNA is extracted with
25 phenol/chloroform/isoamyl alcohol (25:24:1), ethanol precipitated, washed with 80%
45 ethanol, and resuspended in 1000 μ l of TE buffer, pH 8.

5 C. DNA Sequence Transfer:

10 Cloning the PCR amplified product into the acceptor vector: A combination of 4 μ l (40ng) of the topoisomerase treated PCR product (400bp - 2000bp) and 1 μ l (30ng) of the prepared acceptor vector is prepared and incubated at
5 25°C for 5 min., the reaction is placed on ice, and then 1 μ l of the combination is transformed into competent *E. coli* using either chemical transformation or
15 electroporation techniques (Ausubel et. al, Section 1.8). Cells containing the acceptor vectors plus transfer sequence are selected by plating on antibiotic media requiring a resistance marker specific to the acceptor vector. Plates are incubated at 37°C for 12-
20 18 hrs. and resulting colonies are screened by miniprep and restriction digest (Ausubel et. al, Sections 1.6 and 3.1) to identify acceptor vector clones containing the desired transfer sequence.

25 While the foregoing has been with reference to particular embodiments of the invention, it will be appreciated by those skilled in the art that changes in these
15 embodiments may be made without departing from the principles and spirit of the invention, the scope of which is defined by the appended claims.

Claims

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That which is claimed is:

1. A cell-free subcloning system comprising:
 - a donor vector comprising a transfer sequence flanked by site-specific recombination sequences,
 - an acceptor vector comprising a site-specific recombination sequence that matches the site-specific recombination sequences of the donor vector, and
 - a site-specific recombinase capable of recognizing the site-specific recombination sequence.
2. A cell-free subcloning system according to claim 1 wherein the site-specific recombination sequence is recognized by a type I topoisomerase.
3. A cell-free subcloning system according to claim 1 wherein the site-specific recombination sequence is recognized by vaccinia DNA topoisomerase, Cre, Flp, HK022 integrase or lambda integrase.
4. A cell-free subcloning system according to claim 1 wherein the site-specific recombination sequences is identical in the donor and acceptor vectors.
5. A cell-free subcloning system according to claim 1 wherein the site-specific recombination sequence is loxP, loxP511, loxB, loxC2, loxL, loxR, loxΔ117, FRT, Dif, and Att.
6. A cell-free subcloning system according to claim 3 wherein the site-specific recombination sequence is 5'-(C/T)CCTT↓, (SEQ ID NO: 1), 5'-ATAACTTCGTATA GCATACAT TATACGAAGTTAT-, (SEQ ID NO: 4), 5'-GAAGTTCCTATAC TTCTAGAA GAATAGGAACTTC, (SEQ ID NO: 7), 5'-CAAGTT, (SEQ ID NO: 12), or 5'-AACCTT, SEQ ID NO: 13).

5 7. A cell-free subcloning system according to claim 1 wherein the transfer sequence is an EST fragment, a gene sequence, or a coding sequence.

10 8. A cell-free subcloning system according to claim 1 wherein the donor vector and/or the acceptor vector additionally comprise one or more nucleic acid
5 sequences selected from a promoter-enhancer sequence, a selection marker sequence, an origin of replication, or a fusion protein producing sequence.

15 9. A cell-free subcloning system according to claim 6 wherein the fusion protein producing sequence comprises an epitope-tag encoding sequence, an affinity purification-tag encoding sequence, or a functional protein encoding sequence.

20 10. A method of rapidly subcloning a nucleic acid sequence, said method comprising contacting a site-specific recombinase and a cell-free solution comprising
25 a donor vector comprising a transfer sequence flanked by a site-specific recombination sequence recognized by the recombinase, and an acceptor vector comprising at least one site-specific recombination sequence recognized by the
15 recombinase, under conditions suitable to promote the transfer of the transfer sequence from the donor vector to the acceptor vector.

30 11. A method according to claim 8 wherein each site-specific recombination sequence is recognized by a type I topoisomerase.

35 12. A method according to claim 8 wherein the site specific recombination
20 sequences are identical.

40 13. A method according to claim 8 wherein the site-specific recombination sequence is recognized by vaccinia DNA topoisomerase, Cre, Flp, HK022 integrase or
45 lambda integrase.

- 5 14. A method according to claim 8 wherein the site-specific recombination
sequence is 5'-(C/T)CCTT↓, (SEQ ID NO: 1),
10 5'-ATAACTTCGTATA GCATACAT TATACGAAGTTAT-, (SEQ ID NO: 4),
5'-GAAGTTCCTATAC TTCTAGAA GAATAGGAACTTC, (SEQ ID NO: 7),
5 5'-CAAGTT, (SEQ ID NO: 12), or
5'-AACCTT, (SEQ ID NO: 13).
- 15 15. A method according to claim 8 wherein the transfer sequence is an
EST fragment, a gene sequence, or a coding sequence.
- 20 16. A method according to claim 8 wherein the donor vector and/or the
10 acceptor vector additionally comprise one or more nucleic acid sequences selected
from a promoter-enhancer sequence, a selection marker sequence, an origin of
replication, or a fusion protein producing sequence.
- 25 17. A method according to claim 13 wherein the fusion protein producing
sequence comprises an epitope-tag encoding sequence, an affinity purification-tag
15 encoding sequence, or a functional protein encoding sequence.
- 30 18. A subcloning kit comprising
one or more vectors, each vector comprising a site-specific
35 recombination sequence and one or more additional nucleic acid sequences,
wherein each vector in the kit comprises the same site-specific recombination
20 sequence, and
a site-specific recombinase that recognizes the site-specific
40 recombination sequence in each vector.
- 45 19. A subcloning kit according to claim 15 wherein the site-specific
recombination sequence is recognized by a type I topoisomerase.
- 25 20. A subcloning kit according to claim 15 wherein the site-specific
recombination sequences are identical in the vectors.
- 50
- 55

5

21. A subcloning kit according to claim 15 wherein the site-specific recombination sequence is recognized by a type I topoisomerase.

10

22. A subcloning kit according to claim 15 wherein the site-specific recombination sequence is recognized by vaccinia DNA topoisomerase, Cre, Flp, HK022 integrase or lambda integrase.

15

23. A subcloning kit according to claim 15 wherein the site-specific recombination sequence is 5'-(C/T)CCTT↓, SEQ ID NO: 1), (5'-ATAACTTCGTATA GCATACAT TATACGAAGTTAT-, SEQ ID NO: 4), (5'-GAAGTTCCTATAC TTCTAGAA GAATAGGAACTTC, SEQ ID NO: 7), 10 5'-CAAGTT, SEQ ID NO: 12), or (5'-AACCTT, SEQ ID NO: 13).

25

24. A subcloning kit according to claim 15 wherein the additional nucleic acid sequences are selected from a promoter-enhancer sequence, a selection marker sequence, an origin of replication, or a fusion protein producing sequence.

30

25. A subcloning kit according to claim 19 wherein the fusion protein producing sequence comprises an epitope-tag encoding sequence, an affinity purification-tag encoding sequence, a functional protein encoding sequence, or a proteolytic cleavage recognition sequence.

35

26. A kit comprising

40

at least one donor vector comprising at least one site specific recombination sequence, a transfer sequence, and a first selectable marker, and

45

at least one acceptor vector comprising at least one site specific recombination sequence, a lethal gene and a second selectable marker.

50

55

Donor

DoubleIOP0

GAGGATTGGG CCGTT [GATC] AAGGAAGAC AATAGCAGC ATGCTGGGA TGGGTGGC TCTATGGCTT
 CTCCTAACC GGGAA CTAG TTCCCTTCTG TTATCGTCG TACGACCCCT ACGCACCCG AGATACCGAA

CTGAGGGGA AGAACCAGC TGGGCTCTA GGGGTATCC CCACGGCCC TGTAGGGCG CATTAGCGC
 GACTCCGCTT TTCTTGGTCG ACCCGAGAT CCCCATAGG GGTGCGCGG ACATCGCCG GTAATTGCG

GGCGGTGTG GTGTTACG GACGGTGAC CGTACACTT GCCAGCGCC TAGCGCCCG TCCTTTGCT
 CCGCCACAC CACCAATGG CGTCGCACTG CGATGTGA CGGTGCGGG ATCGCGGGG AGAAGCGA

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GGTCCGATT TAGTGCCTTA CGGCACCTCG ACCCCAAAA ACTTGATTAG GGTGATGGTT CACGTAGTGG
 CCAAGGCTAA ATCAGGAAT GCCGTGGAGC TGGGGTTTT TGAACATAAT CCACTACCAA GTGCATCACC

GCCATCGCCC TGATAGACGG TTTTTCGCCC TTTGACGTTG GAGTCCACGT TCCTTAATAG TGGACTCTTG
 CGGTAGCGG ACTATCTGCC AAAAGCGGG AACTGCAAC CTCAGGTGCA AGAAATATC ACCTGAGAAC

DoubleIOP0

TTCCAAACTG GAACACACT CAACCCCTT [CTAG] AAGGCTATC TCGGTCT
 AAGGTTTGAC CTTGTTGTA GTTGGGGAA GATC TTCCCGATAG AGCCAGA

Figure 1A

Acceptor

	Double TOPO	Double TOPO
<u>AAAAAGCTCC</u>	<u>CCCTTCTATCAAGGG</u>	<u>CGGGAGCTTG</u>
<u>TTTTTCGAGG</u>	<u>GGGAAGTAGTTCCC</u>	<u>GCCCTCGAAC</u>
		<u>GGGAAGATCTTCCC</u>
		<u>ATATAGGTAA</u>

Figure 1B

Recombinant

DoubletOP0

ABAAAGCTCC CCCTTGATC AAGGGAAGAC AATAGCAGGC ATGCTGGGGA TCCGCTGGGC TCTATGGCTT
TTTTTCGAGG GGGAACCTAG TTCCTTCTG TTATCGTCCG TACGACCCCT ACGCCACCCG AGATACCGAA
 CTGAGCGCGG AAGAACAGC TGGGGCTCTA GGGGGTATCC CCACGGCCGC TGTAGCGGCG CATTAAGCGC
 GACTCCGCC TTTCTGGTCC ACCCCGAGAT CCCCCTATAGG GGTGCGCGGG ACATCGCCGC GTAAATTCGCG
 GCGGGGTGT GTGGTTACGC GCAGCGTGAC CGCTACACTT GCCAGCGCCC TAGCGCCCGC TCCTTTTCGCT
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 GGTTCGGATT TAGTGCTTTA CGGCACCTCG ACCCCAAAAA ACTTGATTAG GGTGATGGTT CACGTAGTGG
 CCAAGGCTAA ATCAGGAAAT GCCGTGGAGC TGGGTTTTT TGAACATAAT CCACTACCAA GTGCATCACC
 GCCATCGCCC TGATAGACGG TTTTTCGCCC TTGACGTTG GAGTCCACGT TCTTTAATAG TGGACTCTTG
 CGGTAGCGGG ACTATCTGCC AAAAAGCGGG AACTTGCAAC CTCAGGTGCA AGAAATATATC ACCTGAGAAC

DoubletOP0

TTCCAAACTG GAACAACACT CAACCCCTTCTAGAGGG TATATCCATT
 AAGGTTTGAC CTTGTTGTGA GTTGGGGGAA GATCTTCCC ATATAGGTAA

Figure 1C

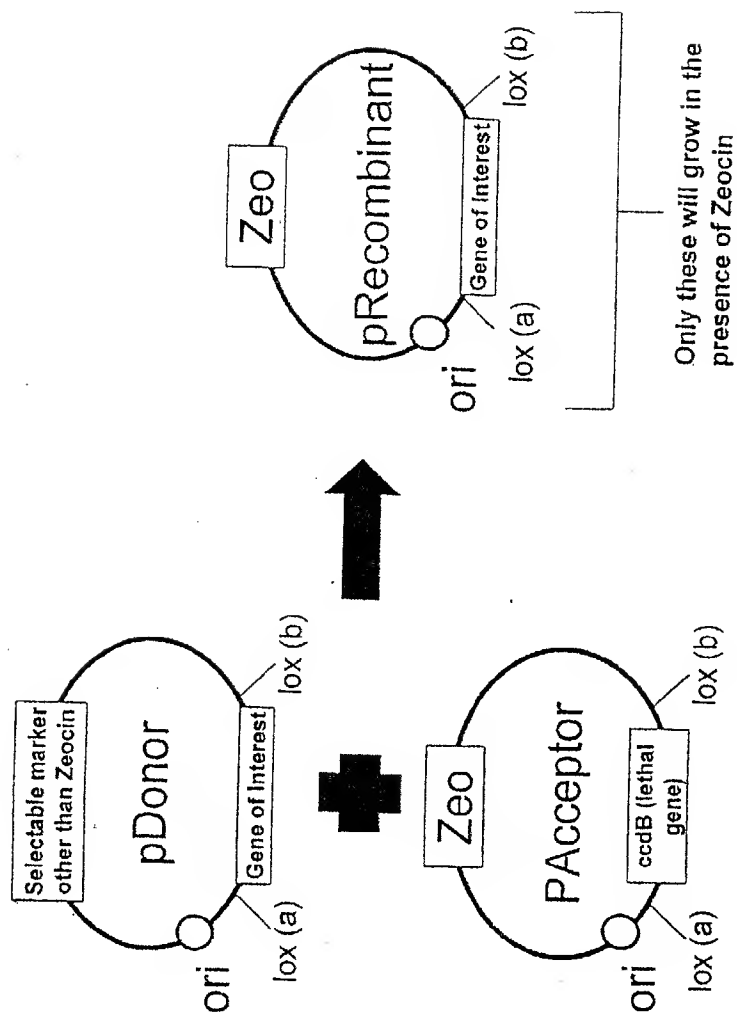


Figure 2

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Marcil, Bob
McConnell, Gina

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WO 00/12687

PCT/US99/19413

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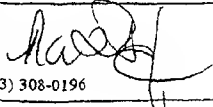
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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/19413

A. CLASSIFICATION OF SUBJECT MATTER IPC(7) : C12N 15/00, 15/64, 15/66; C12P 19/34, 21/06; C12Q 1/68 US CL : 435/6, 69.1, 91.41, 91.42, 91.5, 91.52, 320.1 According to International Patent Classification (IPC) or to both national classification and IPC														
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) U.S. : 435/6, 69.1, 91.41, 91.42, 91.5, 91.52, 320.1 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched NONE Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) Please See Extra Sheet.														
C. DOCUMENTS CONSIDERED TO BE RELEVANT														
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.												
X, P	US 5,888,732 A (HARTLEY et al.) 30 March 1999, see entire document.	1-26												
Y	US 5,766,891 A (S. SHUMAN) 16 June 1998, see entire document.	1-26												
Y	HOESS et al. P1 site-specific recombination: Nucleotide sequence of the recombining sites. Proceedings of the National Academy of Sciences, USA. June 1982, Vol. 79, pages 3398-3402, see entire document.	1-26												
Y	ABREMSKI et al. Bacteriophage P1 Cre-loxP site-specific recombination. The Journal of Biological Chemistry. 05 January 1986, Vol. 261, No. 1, pages 391-396, see entire document.	1-26												
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.														
<table border="0"> <tr> <td>* Special categories of cited documents:</td> <td>* T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</td> </tr> <tr> <td>* A* document defining the general state of the art which is not considered to be of particular relevance</td> <td>* X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</td> </tr> <tr> <td>* E* earlier document published on or after the international filing date</td> <td>* Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</td> </tr> <tr> <td>* I* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</td> <td>* G* document member of the same patent family</td> </tr> <tr> <td>* O* document referring to an oral disclosure, use, exhibition or other means</td> <td></td> </tr> <tr> <td>* P* document published prior to the international filing date but later than the priority date claimed</td> <td></td> </tr> </table>			* Special categories of cited documents:	* T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention	* A* document defining the general state of the art which is not considered to be of particular relevance	* X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone	* E* earlier document published on or after the international filing date	* Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art	* I* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	* G* document member of the same patent family	* O* document referring to an oral disclosure, use, exhibition or other means		* P* document published prior to the international filing date but later than the priority date claimed	
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* A* document defining the general state of the art which is not considered to be of particular relevance	* X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone													
* E* earlier document published on or after the international filing date	* Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art													
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* P* document published prior to the international filing date but later than the priority date claimed														
Date of the actual completion of the international search 10 DECEMBER 1999		Date of mailing of the international search report 27 JAN 2000												
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230		Authorized officer IREM YUCEL  Telephone No. (703) 308-0196												

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/19413

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	NASH et al. Heteroduplex substrates for bacteriophage lambda site-specific recombination: cleavage and strand transfer products. The EMBO Journal. 1989, Vol. 8, No. 11, pages 3523-3533, see entire document.	1-26
Y	SHUMAN, S. Recombination mediated by vaccinia virus DNA topoisomerase I in <i>Escherichia coli</i> is sequence specific. Proceedings of the National Academy of Sciences, U.S.A. November 1991, Vol. 88, pages 10104-10108, see entire document.	1-26

INTERNATIONAL SEARCH REPORT

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B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used).

USPATENTS, DERWENT, STN-CAPLUS, DIALOG, MEDLINE, BIOSIS, SCISEARCH

terms: cell-free, in vitro, recombinase?, clon?, subclon?, Cre, lnx?, att?, site, specific, integrase?, topoisomerase?, lethal, toxic, fusion, protein?, polypeptide?, kit?, donor?